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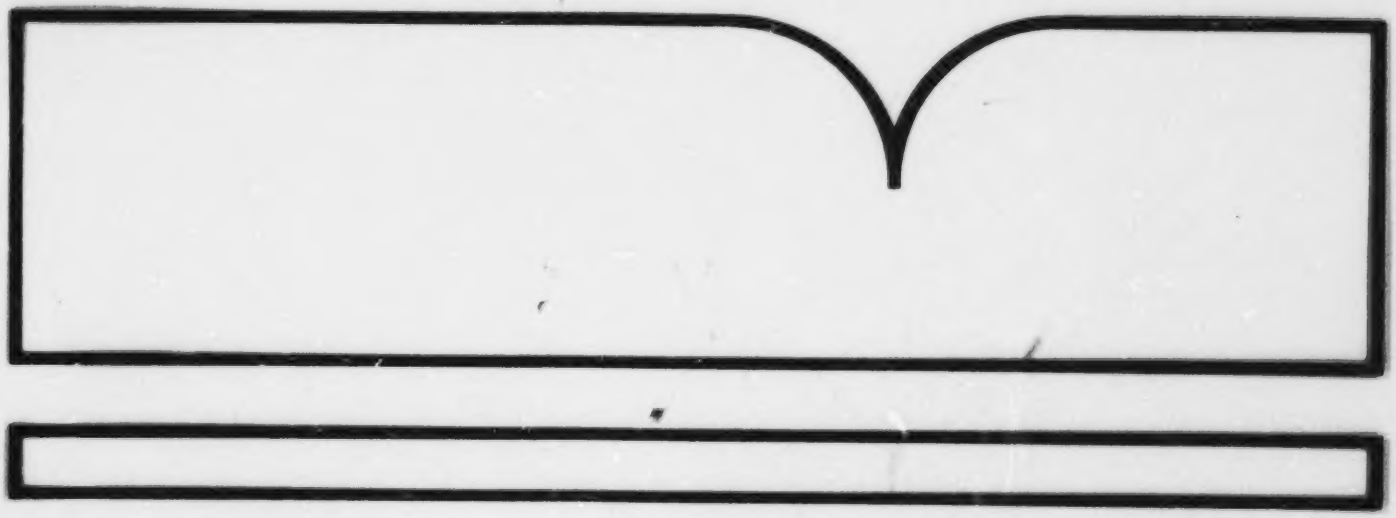

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16. ABSTRACT The search for rapid methods in sanitary bacteriology is more urgent today than ever before because of increased necessity for processing poorer quality source waters and controlling quality of sewage effluent discharges. Selection of criteria for rapid tests involving either modified conventional procedures or those that require special reagents and instrumentation must consider quantitative or qualitative results within a few hours, data of acceptable sensitivity, selectivity, and precision, and procedures that are amenable to average laboratory operations. The increased cost per test compared to conventional monitoring may be justified in terms or protection afforded to public health. The most promising candidate methods include: use of metabolic inducer compounds, radioisotope labeled substrates, specifically labeled fluorescent antisera and organism specific genetic probes.

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1. INTRODUCTION

With the evolution of treatment processes to control and improve the microbial quality of drinking water, wastewaters and other infectious wastes, plant engineers have long sought operational monitoring methods that would provide a rapid characterization of the product water for determining treatment effectiveness. The increase in pollutional loading of receiving waters and the need to regulate microbial quality of effluents and water supplies have intensified the interest in rapid methods. Specifications for a practical rapid analysis of water in the context of this paper require that the method (a) achieves the quantitative measurement within a few hours; (b) provides data that correlates with standard procedures; (c) is relatively uncomplicated so the average lab technician can reliably perform the test; and (d) is cost effective for benefits achieved.

Treatment modifications to reduce dissolved organic content and the use of alternative disinfectants to minimize reaction products in drinking water necessitate more careful monitoring of microbial barriers. Elimination of prechlorination, use of carbon adsorbers, or rapid conversion of free chlorine to chloramines may result in penetration of organisms further into the treatment process chain. This undesirable event places greater reliance on final disinfection in preventing entry of microorganisms into the distribution network. Water quality in distribution must also be protected from contamination and rapid monitoring to detect such occurrences is also desirable.

Wastewater treatment is not a precisely controlled process, partly because of wide variations in the nature of municipal sewages, impact of stormwater runoff on treatment capacity, and operator skill. Efforts to compensate for the variations in BOD and microbial content in treated effluents generally have been directed toward excessive, wasteful applications of chlorine to "protect" downstream users of receiving waters. This approach to treatment has resulted in adverse effects on aquatic life, aftergrowth of bacteria downstream, and formation of chloroganics.

Where downstream uses involve shellfish growing waters, recreation, or a water supply intake, monitoring the microbial quality of the water through a rapid technique could provide a crucial first-alert in preventing the occurrence of public health problems.

First alert capability demands the application of rapid microbiological characterization to yield information on abrupt changes in water quality that could increase the public health risk to users of the water resource. Once a rapid detection procedure is developed for an indicator or pathogen, the logical progression is to integrate

sample collection and analysis in an automated mode to provide more frequent information on water supply or wastewater treatment process effectiveness. Careful analysis of this data would clarify quality trends and provide more precise information on process control to improve product water. Such information would also be of singular value as an aid in restoring quality of drinking water during excessive challenge, treatment failures, contamination introduced into distribution water through line breaks and cross connections.

1.1. Specifications For Rapid Methods

Rapid methods for environmental bacteriology must provide specificity, sensitivity and precision. Their reliability should be capable of documentation with quality control procedures. In general, non-specific detection of microorganisms has limited application in drinking water because many heterotrophic organisms encountered are of no apparent health significance. However, if performance information on disinfection processes is desired, then non-specific detection of general microbial activity is warranted.

Health significance of water quality is traditionally determined by testing water for organisms or groups of organisms common to the fecal flora of warm-blooded animals. The most common indicator groups include a broad spectrum of genera or species such as total coliform and fecal streptococcus. Other indicators comprise a narrower grouping of related organisms typified by fecal coliform (fecal coli), enterococcus, Bacteroides, and sulfate-reducing clostridia. Individual species of bacteria (E. coli, Streptococcus fecalis, and Clostridium perfringens) have been recommended as more specific indicators of fecal contamination; in the case of Clostridium perfringens, possible as a more sensitive indicator of the recency

of contamination. In practical terms, speciation of bacterial isolates using multiple-biochemical test systems has not been applied to routine monitoring because the identifications are time consuming and labor intensive. Recent research in immunology and recombinant DNA techniques may provide the means for rapid species identification.

Overall, there is general agreement that treated drinking water may only infrequently contain an occasionally total coliform provided they are not fecal in origin. Thus, rapid tests for total coliforms must be based upon detection of one coliform per 100 ml and species other than E. coli. Species of the total coliform group that have been found most frequently in situations of positive sample violations include Enterobacter aerogenes, Enter. cloaceae, Enter. agglomerans, Citrobacter freundii, Klebsiella pneumoniae, K. oxytoca and E. coli. The characteristic common to all of the species of bacteria included in the total coliform group is the ability to ferment lactose at 35°C. For the total coliform group detection sensitivity at low densities per 100 ml and their accurate quantitation present major challenges to every proposed rapid method. Thus, rapid test concepts for the coliform group are likely to use a common biochemical reaction rather than serological techniques because of antigen antibody cross reactions among related organisms. Serological cross reactivity among the fecal streptococci may also restrict the application of rapid serological procedures for differentiation of this group which includes Streptococcus fecalis, durans, bovis, equinus, and several strain biotypes.

For water uses of greatest public health concern (drinking water, shellfish and recreational waters), water quality standards are established set at values thought to present a minimal risk

of exposure to waterborne pathogens. Of these water uses, drinking water standards are the most stringent requiring that no coliforms be detected in 100 ml of water. For shellfish and recreational water fecal coliform limits of 14, and 200 to 400 organisms per 100 ml, respectively, have been established. The bacterial density limits suggested for source water used in treated public water systems are at the lower end of the water quality scale. For these sources, a maximum of 2,000 fecal coliform or 20,000 total coliform bacteria per 100 ml is recommended to lessen the risk of microbial penetration of the treatment barriers. With these values in mind, and the ease in which the coliform group is reduced even in systems providing marginal treatment it becomes apparent that until a breakthrough occurs in rapid test sensitivity, amplification of the original cell density through concentration or enrichment in a sample will generally be necessary.

1.2 Cost Consideration

A major justification for a rapid microbiological technique is its utility in operational monitoring. While public health is always of first concern, monitoring costs can be a very important consideration. What is a reasonable price for quicker results from bacteriological tests? Recently, the cost for routine coliform analysis performed by a city health laboratory was reported to be $3.32 per test by the membrane filter procedure, or $6.21 per test by the multiple tube procedure (6). A survey of commercial laboratories showed that charges for these two procedures ranged from $8 to $12 for the MF test, and from $14.30 to $18.00 for the MPN test. The same cost factors would apply to analyses of samples from public water supplies, recreational waters, or sewage effluents.

Another approach used information from a survey of the cost of bacteriological monitoring for several water plant operations serving cities with populations ranging from 60,000 to 900,000. The survey data revealed test costs of $15.00 per sample. Of course, monitoring cost will vary with the size of the system and cost per test declines sharply for the laboratory involved in examining more than 60 samples per week. Part of this economy of scale is also a function of the required number of samples examined per month based on population served. Analysis of the price for meeting the bacteriological monitoring requirements mandated by U.S. Federal regulations has been estimated to be only one cent of the customer's cost per 1000 gallons of public water supply (3). With the preceding information in mind, it becomes apparent that a cost increase for a rapid monitoring procedure is justifiable and could approximate twice the cost of routine analyses for the benefits achieved.

Use of a rapid test in an effective monitoring program, including a quick action response when undesirable results are obtained, could be of substantial benefit. It would be particularly advantageous when the risk is due to fluctuating source water quality that challenges existing treatment processes, the direct reuse of high quality wastewater, or exposure to recreational or shellfish growing water. While small water systems may have the greatest difficulties in delivering a continuous supply of safe drinking water, the application of expensive and complicated rapid test monitoring will not be feasible. In these situations, simple, low cost, basic measurement of water quality are more urgently needed to supplement annual sanitary surveys and operator training programs.

2. RAPID TEST CANDIDATES USING CONVENTIONAL PROCEDURES

The ideal rapid method candidate is one that would utilize methods and materials available to every bacteriological laboratory. Among the routine methods, the membrane filter technique offers much promise, allowing concentration of organisms from large sample volumes. Furthermore, membrane filters permit visualization of developing microcolonies, and use of or placement in contact with different media or biochemical substrates without disrupting their growth. Furthermore, colony isolation makes verification procedures easier.

2.1 Rapid Colorimetric Determination

The rapid colorimetric test is based on simple membrane filter concentration of bacteria from a sample followed by staining the trapped bacterial cells with Safranine O dye (19). Excess dye is flushed away and an agent is applied which decolorizes only the dye on the membrane filter, not dye absorbed by bacterial cells. Concentrations of bacterial cells $\geq 10^5$ organisms per ml on the 10mm diameter membrane filter yield a pink to red color; bacterial densities below 10^5 organisms per ml yield a white or slightly yellow filter. The color of the membrane is then compared to a standard color guide, thus providing a semi-quantitative indication of the bacterial density in the sample. Larger water sample portions (10 ml, 100 ml or 1,000 ml) may be tested as necessary to achieve a detection level of 10^5 cells. Where water color interferes, the sample must be passed through an anionic exchange resin to remove color materials. If iron particulates occur, the sample is pretreated with a rust solvent (RoVer, Hach) so that metal oxides do not interfer with bacterial staining or the decolori-

zation of the filter surface. Use of these sample clean-up procedures alone or in combination did not result in significant loss of the semi-quantitative results. Obviously, this rapid test, which can be performed in a few minutes, is restricted to use in industrial cooling waters (18) or other process waters where a general measure of microbial density is adequate and the occurrence of nonviable organisms does not effect the interpretation of results.

2.2 Rapid Membrane Filter Approach

A seven hour fecal coliform test is possible using a lightly buffered lactose medium (M-7h agar) and incubation at 41.5°C (13). Bromcresol purple and phenol red indicators in the medium change to yellow color in the presence of developing fecal coliform colonies. The addition of 0.5 percent d-mannitol permits rapid differentiation of some fecal coliform strains which have an initial preference for utilizing mannitol. Use of 41.5°C rather than 44.5°C for incubation shortened the lag phase and reduced heat stress. Field results using this method correlated well with the 24 hour standard membrane filter fecal coliform test, although stressed indicator strains may produce poor differentiation with only 7 hours incubation.

2.3 Coliphage Detection

The lysis of coliform bacteria by DNA or RNA coliphage to form plaques in a confluent growth lawn of a host culture offers a simple plating procedure that can produce results in 4 to 6 hours. The test concept relies on a positive correlation between the density of coliform bacteria and coliphage in ambient waters and sewage effluents (8, 20). Because coliphage appear to be more disinfectant resistant than coliforms, the quantitative relation-

ship may be distorted when the test is applied to treated drinking water. Successful use of a rapid coliphage measurement is related to selection of a host E. coli culture that is susceptible to infection by the majority of coliphage present in the sample. E. coli C (ATCC No. 13706) appears to satisfy this requirement and is used as a standardized density of approximately 1 x 10^9 cells per ml for developing a lawn of host cells.

3. SPECIAL TECHNIQUES REQUIRING AMPLIFICATION OF BACTERIAL NUMBERS

Efforts to shorten the time required to detect either bacterial indicators or individual species in a water sample flora to less than 5 hours will require the use of special instrumentation and materials. These procedures must be capable of quantitating viable organisms directly or after a brief period of amplification in a selective enrichment. Instrumentation and test materials for these methods are not readily available to the average laboratory and require some training of the laboratory staff, plus an effective quality assurance plan to insure reliability of data. Due to the cost of the associated instrumentation and reagents, application of this category of rapid methods will be limited to large laboratories where resources in expertise and money are more likely to be available.

3.1 Bioluminescence-chemiluminescence

The firefly bioluminescence system is the most studied of the known bioluminescence reactions for rapid detection and quantitation of microorganisms (10-12, 16). Light generated in a living system, or from biochemical components derived from an organism is termed bioluminescence, while light emitted from the reaction of chemicals that are not derived from a biological system is known as chemiluminescence. Using liquid scintillation spectrometry and the firefly

luciferase enzyme system, adenosine triphosphate (ATP) can be detected in standard solutions over a range of 10^{-9} to 10^{-12} moles (16). This system for measuring ATP has been used to measure total microbial biomass in lake sediments (10, 11) and a minimum of 100 to 300 bacteria per ml in spacecraft water supplies (12). Recent studies to establish correlations between the heterotrophic bacterial population of drinking water and the ATP concentration have been unsuccessful because total viable biomass is more encompassing than a measure of the bacterial density in potable water. While this test is specific to viable cells, it will not differentiate indicator organisms from other organisms in the water flora.

Luminescence measurements may also be based on the reaction of luminol with hydrogen peroxide when catalyzed by bacterial catalase or cytochromes. Using this chemiluminescence reaction, light emitted (which is directly proportional to cell density) is sensed by a photometer and recorded. Unfortunately, this approach may respond to soluble porphyrins and oxidizing agents from dead cells as well as viable bacteria.

3.2 Gene Probes for Specific Organisms

Developments in microbial genetics have led to methods that can detect minute quantities of genetic information from specific organisms. Rapid detection of enterotoxigenic E. coli (ETEC) from a mixed flora of a contaminated water is a promising example of gene probe application (5). A water sample is processed by membrane filtration with incubation on M-FC medium (anaerobically at 35°C rather than 44.5°C to avoid loss of heat sensitive plasmids coding for endotoxin) for 24 hours. The bacterial colonies on the membrane filter surface are then treated to remove non-DNA components of the cells. The

residual cell constitutents are treated to denature the DNA, separating the two strands in the double helix. The single stranded DNA is held in place on the membrane filter and exposed to a large excess of specific probe DNA. Upon incubation of the probe DNA with the DNA released from bacterial cells on the membrane filter, hybridization of probe DNA with complementary sample DNA may occur. Excess unbound probe DNA is washed away after the required incubation period. Labeling the probe DNA with a radioisotope, enzyme, fluorescent or chemiluminescent molecule permits detection of reaction. Except for radioisotopic labels requiring autoradiography. These assays can be completed in a few hours and are at least 1000 fold more sensitive than serological tests.

3.3 Specific Enzyme Assays

Specific enzyme detection offers another approach to differential detection of viable bacteria from a sample, provided there are sufficient cells present to provide measurable enzyme concentrations. B-galactosidase and formic hydrogen lyase are inducible enzyme systems of coliform that are associated with lactose fermentation (4). Unfortunately, B-galactosidase is produced not only by E. coli but also by Shigella, Proteus rettgeri, P. hauseri and Clostridium species (9). For fecal coliform measurement, biosynthesis of glutamic acid decarboxylase can be used as a detection marker and is directly proportional to fecal coliform concentration. The glutamate decarboxylase enzyme measurement is coupled to use of a hollow fiber ultrafiltration system to concentrate the coliforms from a sample (17). Based on the concentration of enzyme detected, the density of fecal coliforms per sample volume can be estimated. The sensitivity of this test requires 5×10^4 E. coli per milliter, a concentration that would indicate grossly polluted water. For water samples with

very low fecal coliform members, amplification by enrichment culture could achieve the minimum cell concentration needed, but would require an extended time period. For instance, one coliform in 100 ml of sample when enriched in lactose broth at 41°C for 11 hours would yield a positive glutamate decarboxylase test (9). An automated system for glutamate decarboxylase detection has been developed which permits continuous or programmed sampling passage through a hollow fiber ultrafiltration system for amplification of fecal coliforms concentrated from 10 to 20 liters, and analysis is completed within 12 to 13 hours. While the automated aspects of the test are excellent, the time frame for analysis falls outside our definition of a rapid test.

3.4 Impedance Measurements

Microbial degradation of carbohydrates in a nutrient medium convert these non-electrolyte materials into a variety of electrically charged molecules and ions. The rapidity of the change in conductivity, measured as a change in electrical impedance to an alternating current passed through the culture, is directly related to the density of bacteria in a specific medium over a defined time period (2). A minimum bacterial density, generally 10^6 to 10^7 cells per ml, is essential to achieving a detectable impedance signal (2, 15). With high quality water samples a lag period will occur with no detectable change in the impedance signal until the target organism density reaches the threshold detection level. Since the length of the amplification period is inversely proportional to the original viable cell density in the water sample, a standard curve developed from a 10-fold dilution series of a pure culture of a predominant indicator group organism is used to estimate the unknown bacterial

density in the original sample. The base line impedance value is determined by inclusion of a sterile medium control for comparison with the test sample.

Careful selection of the test medium and incubation temperature is important since many species of bacteria, yeast and fungi growing in various media can produce a detectable impedance signal. For fecal coliform detection, specificity and minimal lag phase can be achieved by use of lactose fermentation at an elevated temperature of 41.5°C. While a lactose based medium and incubation at 35°C should optimize recovery of total coliforms. Some success in rapid detection of heterotrophic bacteria has been achieved with the standard plate count medium, investigators need to explore the potential for improved recoveries that may be achieved with use of m-SPC and R2A medium at 28°C (1).

Since impedance measurement is time dependent and a minimum concentration of 10^6 to 10^7 cells per ml is needed to generate a change in impedance, application of this rapid test is limited to coliform monitoring of sewage effluent laden receiving waters used as source water for public water supply, or recreational bathing areas. The use of the rapid impedance test for coliform detection in drinking water is difficult because the detection level is one coliform per 100 ml. An amplification period of 18 hours or longer would be required to achieve this detection level. This test sensitivity might be possible using one liter sample volumes combined with membrane filter concentration of low densities of coliforms and incubation of the membrane filters in a selective test medium. The coliform density would still be correlated to detection time through the use of standard curves for predominant coliform

species. Instrumentation is available which is adaptable to automation and multiple sample analyses to provide frequent monitoring of the quality of a raw water or sewage.

3.5 Radiometric Methods

The basis for all radiometric bacterial detection methods involves the use of a radioactively tagged substrate that is either incorporated into the bacteria to permit detection, or metabolized by the bacteria with release of a radioactively tagged end product that can be detected, quantitated and correlated with cell density. While isotope incorporation permits easy quantitation, specificity for water quality indicators in a mixed flora is not practical using radicactive phosphorus or sulfur. Furthermore, the short half-lives of these isotopes may present practical difficulties and the relatively high energy Beta particles emitted present a health hazard to laboratory personnel.

The use of a ^{14}C-labeled substrate in a fermentation reaction can be used to differentiate coliforms from the mixed bacterial flora of a water sample. Basically, the radioactive labeled fermentable sugar, or related metabolite is placed in the medium, a measured water sample is added, and the inoculated medium is incubated at a selective temperature to minimize interference and accelerate growth (14). Radioactive $^{14}CO_2$ from the fermentation is collected by precipitation as $Ba^{14}CO_3$ on filter paper moistened with a saturated solution of barium hydroxide placed in the culture vial. The activity (counts per minute, CPM) of precipitated $Ba^{14}CO_3$ is measured by a liquid scintillation spectrometer and compared with an uninoculated control for correction of background radioactivity. The net value obtained is then converted into an indicator density by interpolation of standard curves from pure culture strains. As few as 10 bacteria

can be detected in 1.5 to 8 hours, depending on the organisms and whether stationary or agitated incubation is utilized. Detection times for E. coli prepared from pure cultures revealed 1 to 10 cells, 100 cells, and 1000 cells produced measurable activity in 6, 3 and 2 hours, respectively. Results for detection of coliforms in natural samples are in general agreement with these standard curves. For drinking water samples, large sample volumes must be concentrated by membrane filtration to achieve detection sensitivity of less than 1 coliform per 100 ml.

While significant progress has been made toward standardizing the radiometric rapid test protocol, precision and reproducibility need further refinements. The major problem appears to be the need to enhance the metabolic activity of coliforms stressed by characteristics of treated water supply and chlorinated sewage effluents. Extending the test time beyond 4 hours defeats the objective of a rapid procedure. Improved results are more likely to come from formulation of a medium that promotes the repair of damaged enzyme systems, the use of inducer compounds to stimulate earlier production or increased concentration of measurable metabolic products, and the inclusion of a supplemental radioisotope labelled substrate that can be preferentially used by stressed coliforms early in their recovery effort (14). However, control of the abiological release of $^{14}CO_2$, and improved control of the uniformity of radioactive labelling of substrates such as glucose, lactose and mannitol may help improve sensitivity of labeled metabolite based rapid tests.

3.6 Molecular Hydrogen Detection

The production of molecular hydrogen by metabolizing bacteria can be measured electrochemically. A linear relationship can be estab-

lished between bacterial density and length of lag phase before hydrogen ion release occurs (21, 22). The procedure utilizes a platinum electrode and a reference electrode inserted into the test culture with incubation at a temperature appropriate for the organisms sought. Evolution of hydrogen ions during growth causes the development of changes in an electrical potential difference between the electrodes, and the voltage differences can be recorded using a strip chart recorder. Incorporation of a multichannel monitor has permitted analysis of several samples every 80 seconds when desired.

This electrochemical detection concept has also been used in a prototype *in situ* buoy system for monitoring fecal coliform densities at estuarine locations for early warning of pollution spills (7). Data from the positioned buoy was sent by radio signals to a central monitoring location. The prototype design limited the number of sample analyses to 10 before the unit was retrieved and resupplied with new medium. Deployment of the prototype was limited to water temperatures above 8°C because of incubation requirements and the maximum operational time was limited to less than 10 days because of electrical power requirements.

Investigations with natural water samples indicated that detection of 10^2 to 10^3 total coliforms per 100 ml of estuarine or fresh water was possible in 6.2 to 8.5 hrs. Fecal coliform concentrations of 10^2 to 10^3 per 100 ml of estuarine water were detected in 3.5 to 6.5 hrs, and 5.0 to 7.0 hrs in fresh water (21).

4. SUMMARY

The search for rapid methods in sanitary bacteriology is more urgent today than ever before because of increased necessity for processing poorer quality source waters and controlling quality of

sewage effluent discharges. Selection of criteria for rapid tests involving either modified conventional procedures or those that require special reagents and instrumentation must consider quantitative or qualitative results within a few hours, data of acceptable sensitivity, selectivity, and precision, and procedures that are amenable to average laboratory operations. The increased cost per test compared to conventional monitoring may be justified in terms of protection afforded to public health. The most promising candidate methods include: use of metabolic inducer compounds, radioisotope labeled substrates, specifically labeled fluorescent antisera and organism specific genetic probes.

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